Research Article

bFGF promotes adipocyte differentiation in human mesenchymal stem cells derived from embryonic stem cells

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Abstract

In this work we describe the establishment of mesenchymal stem cells (MSCs) derived from embryonic stem cells (ESCs) and the role of bFGF in adipocyte differentiation. The totipotency of ESCs and MSCs was assessed by immunofluorescence staining and RT-PCR of totipotency factors. MSCs were successfully used to induce osteoblasts, chondrocytes and adipocytes. MSCs that differentiated into adipocytes were stimulated with and without bFGF. The OD/DNA (optical density/content of total DNA) and expression levels of the specific adipocyte genes PPARγ2 (peroxisome proliferator activated receptor γ2) and C/EBPs were higher in bFGF cells. Embryonic bodies had a higher adipocyte level compared with cells cultured in plates. These findings indicate that bFGF promotes adipocyte differentiation. MSCs may be useful cells for seeding in tissue engineering and have enormous therapeutic potential for adipose tissue engineering.

Key words: embryonic stem cells; mesenchymal stem cells; fibroblast growth factor; adipocyte differentiation.

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Introduction

Embryonic stem cells (ESCs) are pluripotent stem cells (Thomson *et al.*, 1998) and have the ability to remain undifferentiated and proliferate indefinitely *in vitro*, while maintaining the potential to differentiate into cells of all three embryonic germ layers (Thomson *et al.*, 1995). The capacity for virtually unlimited self-renewal and differentiation in ESCs has opened up the prospect of widespread applications for these cells in biomedical research, including cell differentiation, developmental studies, elucidation of gene function, drug screening and cell therapy (Bishop *et al.*, 2002).

Although ESCs have a strong potential to differentiate, the diretion of differentiation is difficult to control. This problem has been resolved to some extent with the development of mesenchymal stem cells (MSCs). MSCs are multipotent stromal cells that have a high capacity for self-renewal while maintaining multipotency. MSCs therefore have enormous therapeutic potential for tissue repair. MSCs have the capacity to differentiate into multiple cell types (Nardi and da Silva Meirelles, 2006), including osteoblasts (Brighton and Hunt, 1991), chondrocytes (Brigh-

ton and Hunt, 1997) and adipocytes. In theory, this capacity can be used to generate any cell, tissue or even organ (Rippon and Bishop, 2004), a characteristic that could be useful in the treatment of human disease. However, there are still problems related to the use of MSCs, such as poor homology, inefficient proliferation and differentiation, and long culture times (Gonda *et al.*, 2008; Rodeheffer *et al.*, 2008; Schipper *et al.*, 2008; De Rosa *et al.*, 2009; Lund *et al.*, 2009). In addition, MSCs may cause serious translocation osteogenesis (Chen *et al.*, 2009) during the treatment of diseases.

The difficulties associated with the surgical treatment of trauma, burns, tumor resection, congenital malformations and other causes of soft tissue defects are well-known. With increased development of adipose tissue engineering, new ideas have been developed in relation to the treatment of soft tissue defects and expansion. Adipose-derived stem cells are ideal seed cells for adipose tissue engineering (Rodeheffer *et al.*, 2008). Adipocytes differentiate primarily from MSCs through the embryonic body (EB) with the help of induction factors (Dani *et al.*, 1997). But long induction times and a low differentiation efficiency are still major problems. Basic fibroblast growth factor (bFGF) is one of the most effective induction factors in cellular differentiation and promotes adipocyte differentiation through activation of Ras-MAP *in vivo* (Hutley *et al.*, 2004; Newell

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et al., 2006). Whether there is a similar effect on ESC differentiation remains to be determined.

In this study, human ESCs were obtained and used to generate MSCs. The totipotency of these cells was assessed. MSCs were induced to differentiate into osteoblasts, chondrocytes and adipocytes. bFGF was used to induce adipocytes from MSCs in a variety of conditions. Finally, the OD/DNA (optical density/content of total DNA) values and the expression level of specific adipocyte genes were measured. Our findings indicate that bFGF can promote adipocyte differentiation from MSCs and that the latter cells could be of enormous therapeutic potential for adipose tissue engineering.

Materials and Methods

Cell culture

An undifferentiated NIH-registered human ESCS H9 cell line (a gift from the National University of Singapore) was cultured on mitotically inactivated mouse embryonic fibroblasts. Briefly, the head and extremities were removed from 12.5-13.5d C57B6 mouse fetuses and fibrocytes were generated from the remains after digestion with 0.25% pancreatin. The cells were passaged when they reached 80% confluence and then cryopreserved in liquid nitrogen. Feeder layer cells were obtained by treatment with 1% mitomycin C for 30 min. When differentiation occurred at the clone center, mechanical isolation was used to passage undifferentiated ESCs to a new feeder layer within a 5-7-day period. The medium used was Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 20% serum (SR, Gibco), 1 mM glutamine (Gibco), 1% nonessential amino acids (Gibco) and 0.1% β-mercaptoethanol. The cells were cultured at 37 °C in a 5% CO_2 atmosphere.

ESC identification

ESCs were cultured with and without feeder layer medium. The activity of totipotency factors Oct-4, SSEA-3 Tra-1-60 and Tra-1-81 was assessed by immunofluorescence staining (Wang and Matise, 2013), while the expression of totipotency factors C-MYC, KLF-4, OCT-4 and SOX-2 was assessed by RT-PCR. The totipotency of ESCs *in vivo* was determined by analyzing teratomas in severe combined immunodeficiency disease (SCID) mice (Shih *et al.*, 2007).

MSCs induction and identification

To induce MSCs, the ESCs in the feeder layer were transferred to a feeder-free system (Xu *et al.*, 2005). After 3-4 days, the cells were digested with dispase, separated and seeded at very low density (10 cells/cm^2) to allow the formation of MSCs (Chen *et al.*, 2009). The ability of MSCs to differentiate was assessed as described elsewhere (Pittenger *et al.*, 1999; Ouyang *et al.*, 2006; Bi *et al.*, 2007). MSCs were induced to osteoblasts, chondrocytes and adipocytes through treatment with osteoblast inducer (β-glycerol phosphate, dexamethasone, ascorbic acid), chondrocyte inducer (TGF-β1) and adipocyte inducer (1-methyl-3-isobutylxanthine, dexamethasone, insulin, indomethacin). The differentiated cells were identified by staining with alkaline phosphatase, Safranin O and Oil Red O.

Adipocyte induction in plate cultures

MSCs were cultured until 80% confluence, after which they were dissociated with pancreatin and cultured in 24-well plates at a density of 5x10^5 cells/mL. After incubation for 24 h to allow attachment, the cells were divided into bFGF-treated (inducer supplemented with 4 ng bFGF/mL, Gibco) and non-bFGF-treated (inducer without bFGF) cells (16 wells/group). One, three, five and seven days after induction four wells for each group were washed three times with phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde. After removal of this solution, the cells were stained with Oil Red O for 30 min and then washed three times with isopropanol. Finally, the cells were suspended in PBS and examined by light microscopy. In addition, OD was measured after the Oil Red O dye solubilized in isopropanol. RNA was extracted from the cells and the specific adipose genes PPARr2 and C/EBPs were detected by RT-PCR. GDPH was used as an internal housekeeping gene (control).

Adipocyte induction in EB

EBs were formed *in vitro*. Briefly, medium was removed from MSCs and then the cells were washed three times with PBS. After treated with pancreatin for 3 min, the cells were dispersed and the overlying solution was removed by centrifugation. The underlying cells were suspended in medium and cultured in hanging upside down plates (2x10^5 cells/mL, 20 μL/EB) at 37 °C. Three days later, the EBs were transferred to new plates and cultured in suspension. A similar experiment in which adipocytes were induced in EBs and then cultured in plates was also done. The EBs were transferred to 24-well plates (five EBs/well) and induced. The EBs were removed on the first, third and seventh days after induction: two EBs were stained with Oil Red O and the remaining three were used to detect specific adipose genes.

Results

Morphology and identification of ESCs

ESCs were cultured with and without feeder layer medium. In both of these conditions, the cells exhibited typical ESCs morphology, *i.e.*, a flat, close, smooth edge and a high nuclear cytoplasmic ratio. However, cells grown in feeder layer medium were thicker and piled up, whereas cells grown without feeder layer medium were flatter and more expanded (Figure 1A,B).
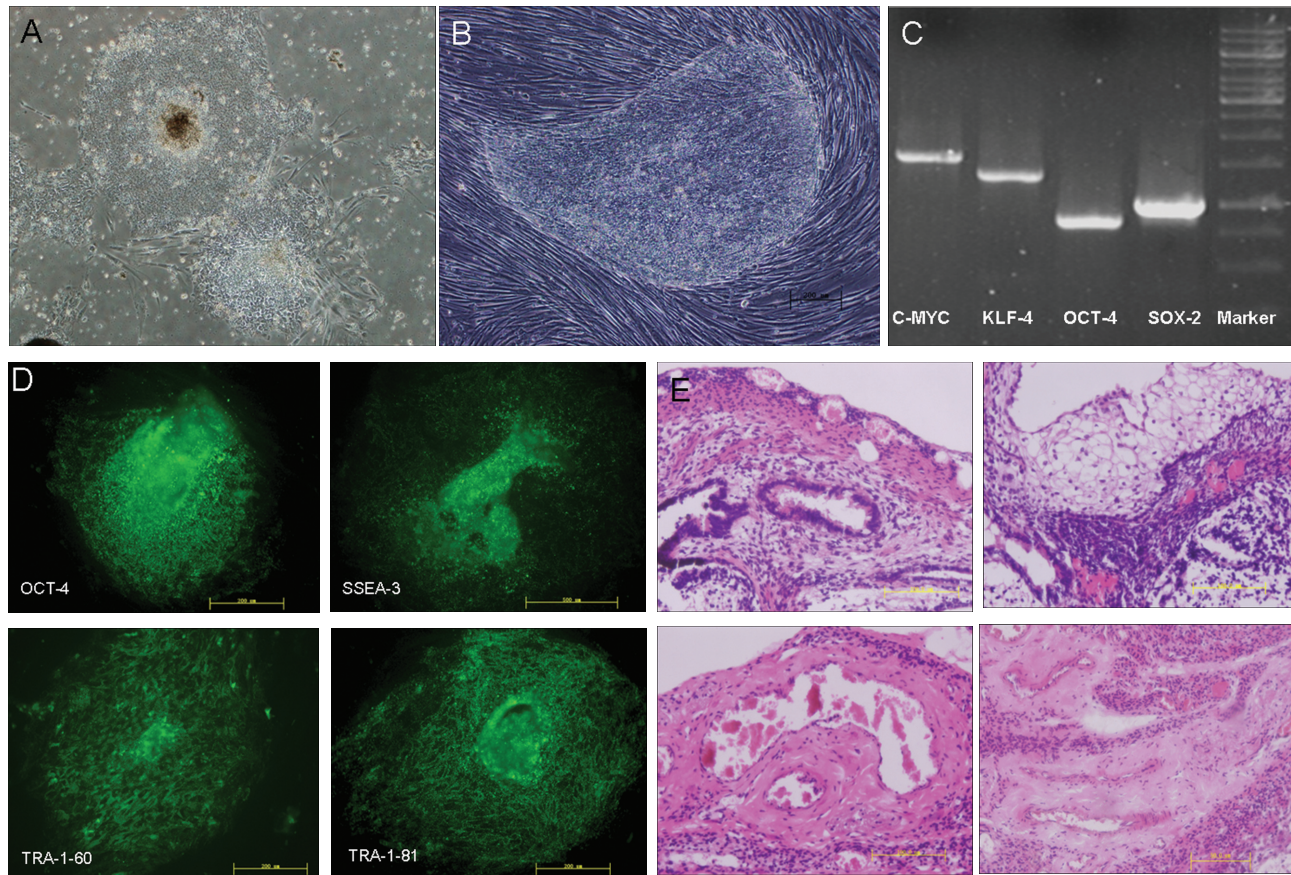
Figure 1 - Morphology and identify of ESCs grown with (A) and without (B) feeder layer medium. (C) RT-PCR analysis of totipotency factors C-MYC, KLF-4, OCT-4 and SOX-2. (D) Immunofluorescence staining of totipotency factors Oct-4, SSEA-3 Tra-1-60 and Tra-1-81 (400x). (E) HE-stained sections of teratomas from SCID mice (400x).

Immunofluorescence staining and RT-PCR of several totipotency factors were used to identify and characterize the ESCs. RT-PCR showed that C-MYC, KLF-4, OCT-4 and SOX-2 were expressed in ESCs (Figure 1C). In addition, OCT-4, SSEA-3, TRA-1-60 and TRA-1-80 were detected by immunofluorescence staining (Figure 1D). These results indicated that these cells were totipotent. The injection of ESC subcutaneously into SCID mice resulted in teratoma formation after eight weeks, as shown by hemotoxylin and eosin staining of tissue sections, thus confirming the totipotency of the cells. As shown in Figure 1E, the teratoma consisted of three germ layers: digestive tract epithelial layer derived from entoderm, adipose tissue layer derived from mesoderm and neuroectodermal tissue layer derived from ectoderm.

Morphology and identification of MSCs

MSCs were derived from ESCs. With continuous passage, the number of heterologous cells decreased while the number of homologous cells gradually increased. After three passages, the cells began to exhibit MSC morphology. ESCs-derived fibroblast colonies were finally formed after at least 13 passages (Figure 2A-C). The ability of

MSCs to differentiate into different cell types was assessed by exposing these cells to various inducers. Three types of cells were induced and identified by staining. Two weeks after induction, osteoblasts were identified by the presence of black mineral calcium deposits (Figure 2D) and four weeks after induction adipocytes showed red (lipid) droplets and chondrocytes were stained watermelon red (Figure 2E,F).

Adipocyte induction in plate cultures

The cells were removed 1, 3, 5 and 7 days after induction, respectively. There was no obvious adipocyte formation in non-bFGF-treated cells, as judged by staining with Oil Red O (Figure 3A-C). In contrast, in bFGF-treated cells, adipocytes appeared after three days and reached 60% confluence after seven days (Figure 3D-F). The level of staining was quantified by measuring the optical density (OD) of Oil Red O in adipocytes and the OD/DNA values of the cells were compared. As shown in Figure 3I, the OD/DNA values of bFGF-treated cells were three times higher than those of the non-bFGF-treated cells. RT-PCR was used to detect the expression of specific adipose genes in these cells. The expression levels of PPARr2 and
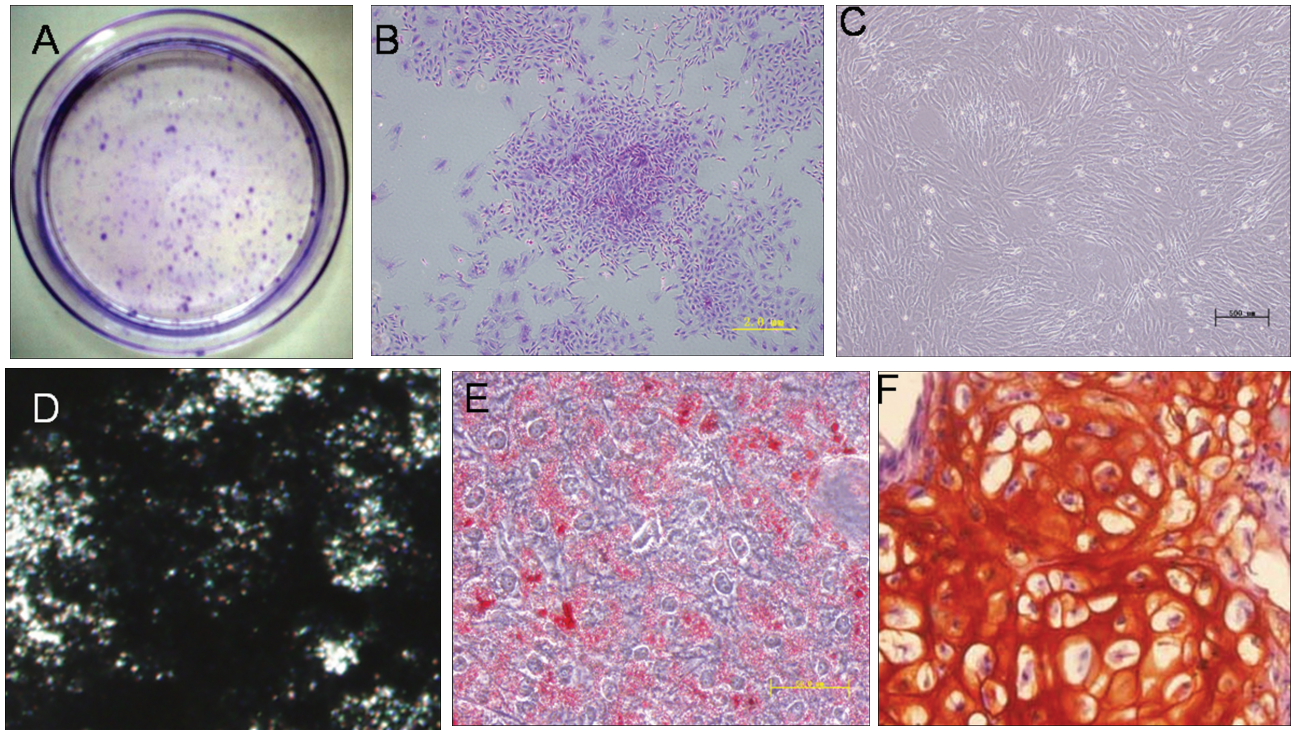
Figure 2 - Morphology and identify of MSCs. (A) Single cells derived from ESCs digested with trypsin. (B, C) Morphology of MSCs after 3 and 13 passages, respectively. (D-F) Stained osteoblasts, adipocytes and chondrocytes, respectively, induced from MSCs.

C/EBPs in bFGF-treated cells were significantly higher than in the control group and increased with the number of passages (Figure 3J-M). These results indicated that the adipogenic rates in bFGF-treated cells were higher than in non-bFGF-treated cells and suggested that bFGF may play an important role in adipocyte differentiation.

Adipocyte induction in EBs

EBs were formed by MSCs at a density of 2 x 10^5 cells/mL (Figure 4A). The ability of EBs to differentiate was assessed by comparing cell induction in plate cultures and EBs. The cells were detected 1, 3 and 7 days after induction. Adipocytes began to grow after one day and three days in EBs and plate cultures, respectively (Figure 4B-G); this growth increased significantly with the number of passages. In addition, the OD/DNA values, as well as the expression levels of PPARr2 and C/EBPs, were significantly higher in EBs than in plate cultures (Figure 4I-K). These results showed that adipocyte induction was easier and greater in EBs.

Discussion

ESCs are pluripotent stem cells with a capacity for indefinite proliferation and a strong potential for differentiation. However, the direction of ESC differentiation is difficult to control. MSCs derived from ESCs are multipotent stromal cells with a high capacity for self-renewal and maintenance of multipotency. MSCs can differentiate into various cell types and are considered to be of major therapeutic potential for tissue repair. Recently, MSC-derived adipocytes have provided a new approach for treating soft tissue defects and expansion. In this context, bFGF is an important inducer of adipocyte differentiation.

In this study, ESCs were cultured on mouse embryonic fibroblasts. These cells can be cultured, passaged and cryopreserved, and retain their proliferative capacity after continuous passage. Several totipotency molecular markers with an important role in maintaining the capacity for self-renewal (Eiges *et al.*, 2001; Richards *et al.*, 2002) occur in these cells, including stage-specific embryonic antigens (SSEA-1, SSEA-3, SSEA-4), transcriptional regulation antigens (TRA-1-60, TRA-1-81), homologous protein transcription factor (Nanog) and transcription factors OCT3\4 and SOX-2. As shown here, three germ layer teratomas were also generated. These results indicate these cells are totipotent and that the method for establishing them is feasible. MSCs were generated from ESCs. With continuous passage, the cells began to form fibroblast colonies. MSCs can proliferate indefinitely while retaining their potential for differentiation, as shown by the identification of totipotency factors. Indeed, MSCs were totipotent and could differentiate into osteoblasts, chondrocytes and adipocytes after stimulation with a specific inducer. MSCs can therefore be regarded as seed cells for tissue engineering. The relatively controllable differentiation may provide a new approach for adipose tissue engineering.
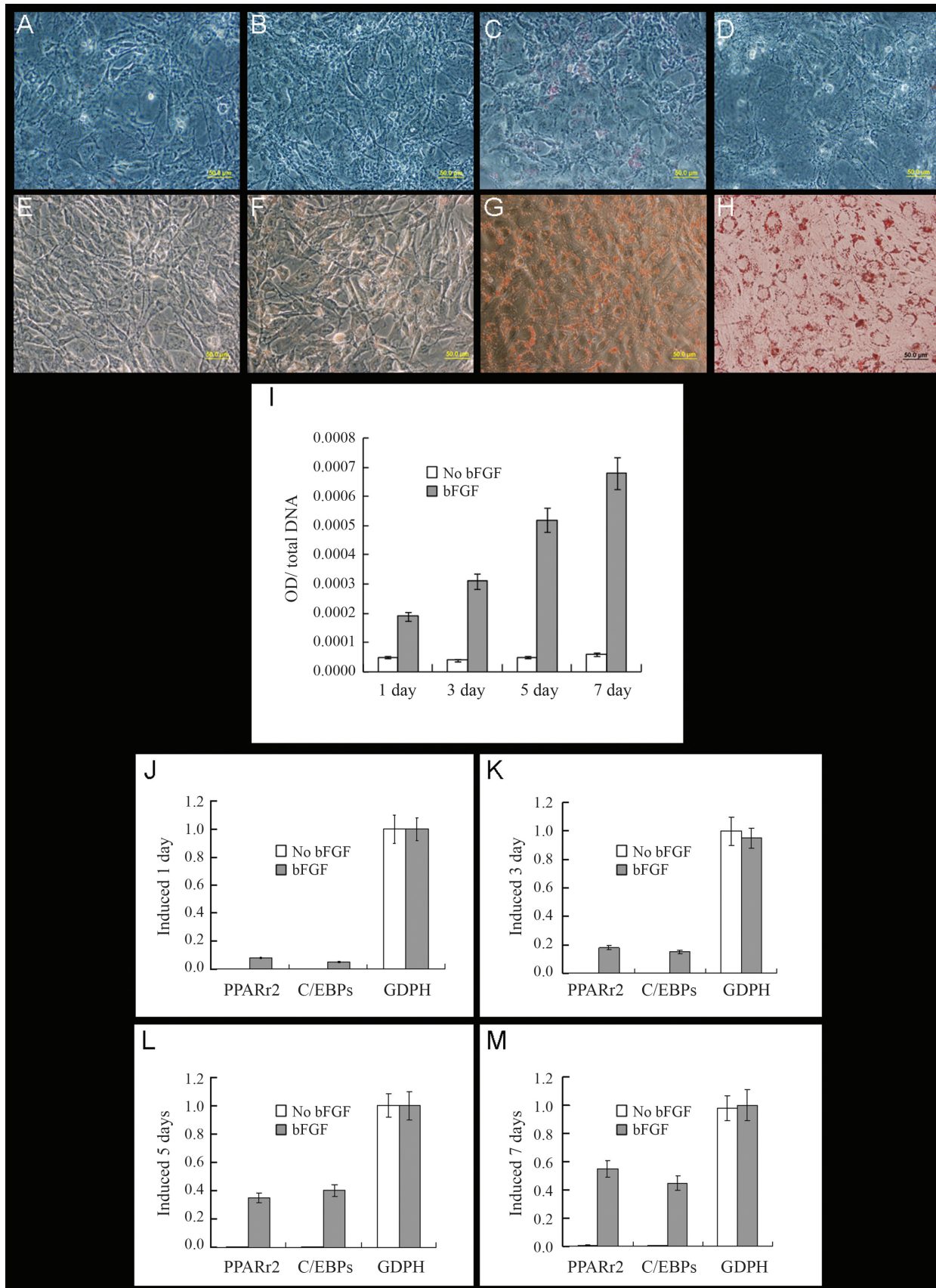
Figure 3 - Adipocyte induction in plate cultures after 1, 3, 5 and 7 days in the absence (control group; A-D, respectively) and presence (E-H, respectively) of bFGF. (I) OD/DNA values and (J-M) RT-PCR analysis of the specific adipocyte genes PPARr2 and C/EBPs 1, 3, 5 and 7 days after induction, respectively.
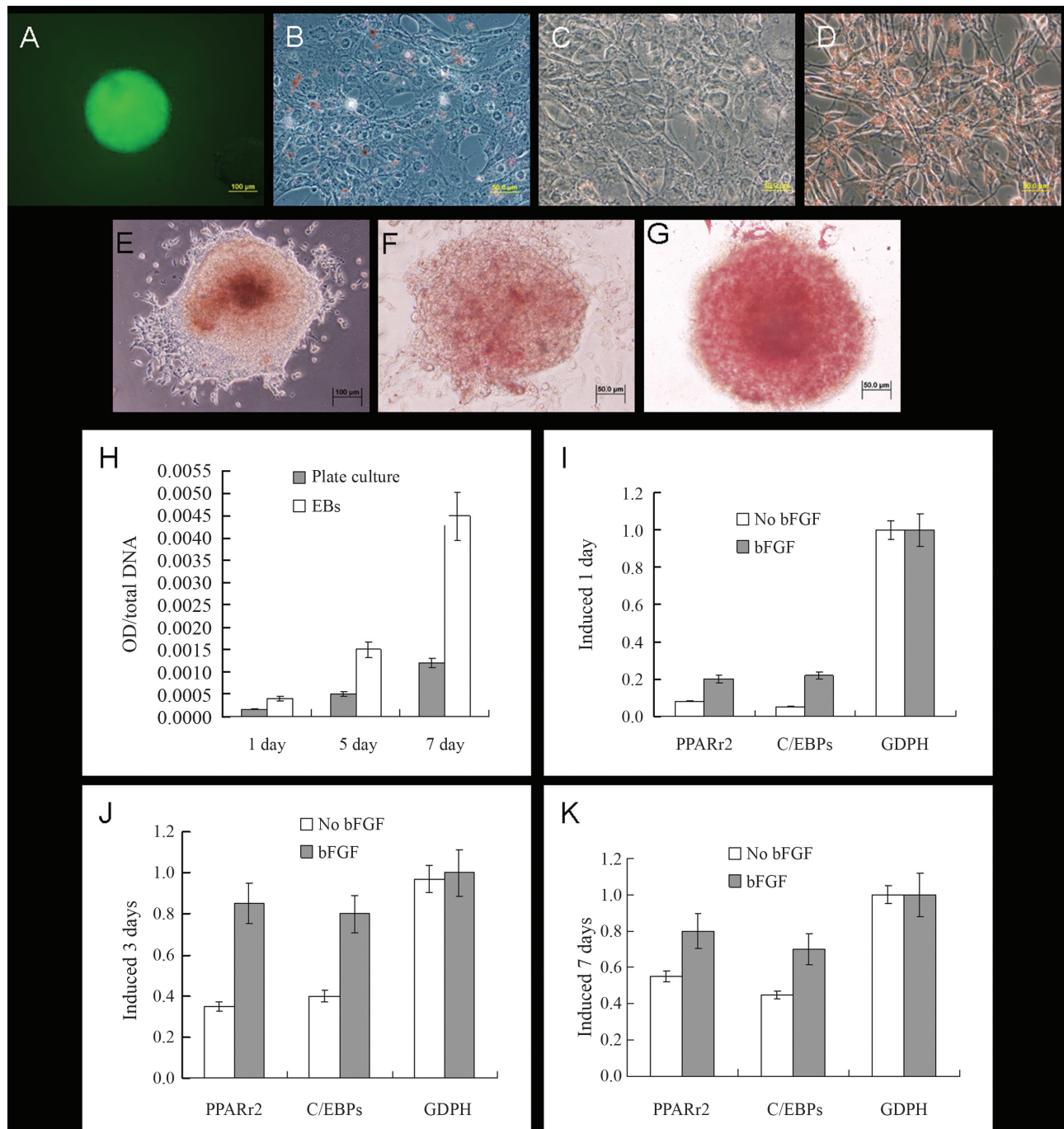
Figure 4 - Adipocyte induction in EBs. (A) EB formed by MECs. (B-D) Adipocytes induced after 1, 3 and 7 days, respectively, in plate cultures. (E-G) Adipocytes induced after 1, 3 and 7 days, respectively, in EBs. (H) OD/DNA values and (I-K) RT-PCR analysis of specific adipose genes PPARr2 and C/EBPs 1, 3 and 7 days after induction, respectively.

FGFs are heparin-binding protein mitogens that stimulate the division of most cell types in culture. During embryonic development, FGFs have diverse roles in regulating cell proliferation, migration and differentiation. In adult organisms, FGFs are homeostatic factors and have important functions in tissue repair and response to injury (Ornitz and Itoh, 2001). FGFs regulate a broad spectrum of biological functions by signaling through FGF receptors. Knock-down of FGF1 expression by small-interfering RNA reduces FGF-1-stimulated signaling events, proliferation and priming (Widberg *et al.*, 2009). The FGF signal pathways include the RAS/MAP kinase, PI3 kinase/AKT and PLCγ pathways, of which the RAS/MAP kinase pathway is the most important (Yun *et al.*, 2010).

Various studies have shown that bFGF promotes adipocyte differentiation. For example, FGF-1 promotes adi-

pogenesis of primary human preadipocytes (phPA) through ERK1/2. ERK1/2 activation is necessary for human adipogenesis in the absence of mitotic clonal expansion. FGF-1 induces robust phosphorylation of ERK1/2 in early differentiation and the inhibition of ERK1/2 activity significantly reduces phPA differentiation (Newell *et al.*, 2006); FGF-2 significantly enhances the differentiation of adipocytes from human adipose-derived stem cells. In cells treated with FGF-2 before adipocyte induction, the mRNA expression of peroxisome proliferator-activated receptor γ2, a key transcription factor in adipogenesis, is unregulated (Kakudo *et al.*, 2007). As shown here, bFGF plays a similar role in ESC differentiation. In bFGF-treated cells, induced adipocytes began to appear after three days and reached 60% confluence after seven days; in contrast, no adipocytes formed in non-bFGF-treated cells. The high OD/DNA values and expression levels of specific adipocyte genes seen here clearly showed that bFGF improved the efficiency and speed of adipocyte differentiation.

Different cellular morphologies may influence the differentiation of MSCs. EBs are formed from cell aggregates in non-adherent spheroids (Höpfl *et al.*, 2004). The molecular and cellular morphogenic signaling and events in EBs recapitulate numerous aspects of embryonic development and, as shown here, result in differentiation of the cells into three embryonic germ layers (endoderm, mesoderm and ectoderm), in a manner similar to that associated with the gastrulation of an epiblast-stage embryo *in vivo* (Itskovitz-Eldor et al., 2000). In this study, EBs were also induced and compared with cells grown in plate cultures. Adipocytes were easier to induce in EBs than in plated cells. This phenomenon provides additional evidence for bFGF function, but also highlights the high differentiation capacity of EBs.

In conclusion, human ESCs and MSCs were successfully generated and shown to be totipotent. bFGF significantly enhanced the differentiation of adipocytes from MSCs, and adipocyte induction was greater in EBs than from MSCs. These results indicate that bFGF promotes adipocyte differentiation and that MSCs may be of enormous therapeutic potential for adipose tissue engineering.

Acknowledgments

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